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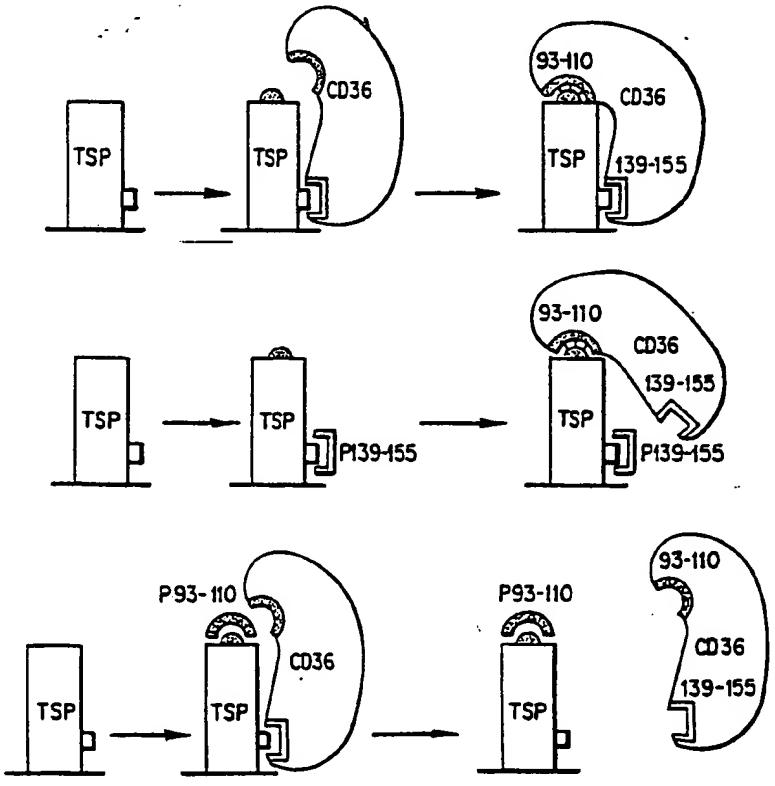
(54) Title: MODULATION OF THROMBOSPONDIN-CD36 INTERACTIONS

(57) Abstract

Ligands are provided which specifically bind to human thrombospondin. Methods are also provided which employ the ligands to modulate the interactions of thrombospondin with platelets and cells bearing receptors for thrombospondin.



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MODULATION OF THROMBOSPONDIN-CD36 INTERACTIONS

This application relates to thrombospondin and the receptor therefor, known as CD36, GPIIIb or GPIV, and to ligands and methods which can be used to either augment or inhibit interactions between thrombospondin and the receptor. This application also relates to platelets and various cells bearing thrombospondin receptors.

BACKGROUND OF THE INVENTION

The specific binding of adhesive proteins to cell surface receptors or to extracellular matrices is an essential element of multiple cell adhesion phenomena such as tissue development, the immune response, vascular hemostasis and inflammation. A number of adhesive proteins, which may be pleiotropic and show significant functional redundancy, are involved in such processes.

One important mechanism in the generation of adhesion specificity is the induction of a high-affinity binding state in the cell surface receptor following cell stimulation. The best characterized examples are induction of fibrinogen-binding function in the $\alpha IIb\beta 3$ (GPIIb-IIIa) integrin on platelets [Phillips *et al.*, *Cell 65*:359 (1991); Du *et al.*, *Cell 65*:409 (1991)] and the capacity for ICAM-1 binding by LFA-1 on T cells [Dustin *et al.*, *Nature 341*:619 (1989)].

There is now evidence that cell adhesion can involve complex conformational changes in the cell surface receptor induced by the ligand. For example, binding of the recognition sequences Arg-Gly-Asp and the gamma carboxyl terminal dodecapeptide of fibrinogen to the $\alpha IIb\beta 3$ integrin induces conformational changes in this receptor, generating a high-affinity fibrinogen-binding state.

Thrombospondin (TSP) is a multi-domain adhesive macromolecule that interacts with a number of proteins, including fibrinogen, fibronectin and collagen [Leung *et al.*, *J. Clin. Invest. 70*:542 (1982); Lahav *et al.*, *Cell 31*:253 (1982)]. TSP is a major platelet α granule protein and is released and expressed on activated platelet surfaces [Phillips *et al.*, *J. Biol. Chem. 255*:11629 (1980)]. By interacting with fibrinogen, TSP serves to reinforce platelet-platelet cohesion and to stabilize platelet aggregates [Leung, *J. Clin. Invest. 74*:1764 (1984)].

TSP is also synthesized by a variety of cell types and is incorporated into cell matrices, where it may modulate cell adhesion, mobility and growth. TSP also supports adhesion of *Plasmodium falciparum*-infected erythrocytes and has been implicated in the pathogenesis of cerebral malaria [Roberts *et al.*, *Nature 318*:64 (1985); Howard *et al.*, *Blood 74*:2603 (1989)].

CD36 [also known as glycoprotein IIIb (GPIIIb) or glycoprotein IV (GPIV)] is an integral membrane protein present on platelets, endothelial cells, monocytes, erythroid precursors, epithelial cells and some tumor lines [Knowles *et al.*, *J. Immunol. 132*:2170 (1984); Asch *et al.*, *J. Clin. Invest. 79*:1054 (1987); Kieffer *et al.*, *Biochem. J. 262*:835 (1989); Greenwalt *et al.*, *Biochemistry 29*:7054 (1990); Catimel *et al.*, *Blood 77*:2649 (1991)]. CD36 is one of the cell surface receptors for TSP, and the CD36-TSP interaction plays a role in mediating platelet-platelet and platelet-monocyte cell adhesion [Asch *et al.*, *supra*; McGregor *et al.*, *J. Biol. Chem. 264*:501 (1989); Silverstein *et al.*, *J. Clin. Invest. 84*:546 (1989)], as well as cell attachment to matrix. In such interactions, TSP acts as a bridge.

A substance that inhibits new blood vessel formation (an angiogenesis inhibitor) has been found to be a fragment or an isoform of TSP [Good *et al.*, *Proc. Natl. Acad. Sci.* USA *87*:6624 (1990)]. TSP, or a particular isoform of it, may therefore play a

role in regulating cell growth by this mechanism. The receptor site for this TSP isoform has not been defined but may be CD36.

The isolation and characterization of CD36 from platelets has been described by McGregor *et al., supra*; Tandon *et al.* [*J. Biol. Chem. 264*:7570 (1989)] and Tsuji *et al.* [*J. Biochem. 100*:1077 (1986)].

In addition to serving as a receptor for TSP, CD36 can also function as a receptor for the adhesion of *Plasmodium falciparum*-infected erythrocytes [Barnwell *et al., J. Immunol. 135*:3494 (1985); Ockenhouse *et al., Science 243*:1469 (1989)]. This interaction is mediated by a CD36 recognition protein which has recently been identified on parasitized cells. CD36 also reacts with OKM5, an anti-CD36 monoclonal antibody which has been reported to inhibit the cell adhesive functions of CD36, including the *in vitro* binding of *Plasmodium falciparum*-infected erythrocytes to monocytes, endothelial cells and C32 melanoma cells (Barnwell *et al., supra*), suggesting that the OKM5 epitope on CD36 is functionally important.

Recently, the sequence Ser-Val-Thr-Cys-Gly has been identified as a cell adhesive motif in TSP having homology to the maralia circumsporozoite protein [Prater *et al., J. Cell Biol. 112*:1031 (1991); Rich *et al., Science 249*:1574 (1990)].

CD36 also binds a serum protein called platelet agglutinating protein p37, which is found in the sera of thrombotic thrombocytopenic purpura patients. Platelet agglutination protein p37 causes platelet agglutination through binding to membrane CD36 [Lian *et al., Thrombosis and Haemostasis 65*:102 (1991)].

Results of recent studies carried out by Trezzini *et al.* [*Immunology 71*:29 (1990)] suggest that CD36 may even act as a signaling mol cule in human monocytes.

Because interactions between TSP and platelets or cells bearing receptors for TSP are involved in many important biological processes and disease states, there is a need for materials and methods to modulate such interactions.

SUMMARY OF THE INVENTION

The present invention fulfills this need by providing ligands which selectively bind to a region of thrombospondin that specifically binds to a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

This invention further provides ligands which selectively bind to a region of thrombospondin, the presence of which region is induced by the binding to thrombospondin of a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

This invention still further provides methods for augmenting thrombospondin-mediated effects comprising contacting thrombospondin in the presence of platelets or cells bearing receptors for thrombospondin with an effective amount of a ligand which selectively binds to a region of thrombospondin that specifically binds to a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

This invention still further provides methods for inhibiting thrombospondin-mediated effects comprising contacting thrombospondin in the presence of platelets or cells bearing receptors for thrombospondin with an effective amount of a ligand which selectively binds to a region of thrombospondin, the presence of which region is induced by the binding to thrombospondin of a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

Pharmaceutical compositions comprising one or more of the ligands and a pharmaceutically acceptable carrier are also provided by this invention.

BRIEF DESCRIPTION OF THE FIGURES

This invention can be more readily understood by reference to the accompanying Figures, in which:

Fig. 1 is a schematic representation of a proposed model of CD36-thrombospondin interaction.

Fig. 2 is a graphical representation of the effects of polypeptides P93-110 and P139-155 on the binding of CD36 to thrombospondin.

Fig. 3 shows platelet aggregation tracings for human platelets treated with various polypeptides plus ADP (A) or collagen (B) and (C).

Fig. 4 shows platelet aggregation tracings for human platelets treated with collagen plus control buffer, antibodies from hybridoma subclone 7AIh, or various amounts of antibodies from subclone 7AIe.

Fig. 5 is a graphical representation of the binding of ^{125}I-P93-110 to thrombospondin, alone and in the presence of various polypeptides.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

As used herein, the term "ligand" means a molecule which specifically binds to human TSP. Preferably, the ligands of the invention are antibodies or linear polypeptides, although cyclic peptides and non-peptide organic compounds that bind to

TSP and exhibit the activities described below are also contemplated by this invention.

The term "region of thrombospondin" is defined herein to mean a localized sequence of amino acids on the surface of human TSP to which a ligand can bind.

The terms "polypeptide" and "peptide" are used interchangeably herein and are intended to mean the same thing.

The term "specifically binds" is defined in the context of this invention to mean the binding of a ligand to human TSP which is mediated by noncovalent short-range interactions, including but not limited to hydrophobic, ionic, hydrogen bonding and van der Waals interactions. Such interactions are determined by specific amino acid sequences and/or organic compound functional groups.

As used herein, the term "selectively binds to a region of thrombospondin" means that the binding of a ligand to human TSP is both specific and localized to a distinct region of the TSP. This definition specifically excludes molecules such as CD36, the binding of which is specific but not selective because they bind to TSP at more than one region.

The term "receptor for thrombospondin" is defined to mean an amino acid sequence on a platelet or cell which specifically binds to human TSP. One such receptor is known as CD36, GPIIIb or GPIV.

Ligands are provided by this invention which can either augment or inhibit TSP-mediated effects.

The ligands of this invention which augment TSP-mediated effects are exemplified by a polypeptide designated P139-155 having an amino acid sequence defined by SEQ ID NO: 1. This polypeptide represents an epitope on CD36

which is specifically recognized by monoclonal antibody OKM5. Since OKM5 is known to inhibit TSP-CD36 interactions, it would be expected that the polypeptide, by binding to TSP, would do so as well. Yet, surprisingly, the binding of P139-155 to TSP has the opposite effect -- it actually augments the interaction between TSP and its receptor.

The inhibitory ligands of the invention are exemplified by a polypeptide designated P93-110 having an amino acid sequence defined by SEQ ID NO: 2. This polypeptide binds to a site on TSP which is distinct from the site to which P139-155 binds. Surprisingly, the binding of P139-155 to TSP induces the appearance of the site to which P93-110 binds, thereby greatly increasing the binding of the latter polypeptide.

A proposed model of the interactions between TSP and CD36 based upon the observed activities of the exemplary polypeptide ligands of the invention is shown in Fig. 1, although whether this model is correct or not is not essential to this invention.

Panel A of Fig. 1 shows that CD36 is believed to contain two regions which can bind to TSP. The binding of one of these regions (represented by polypeptide P139-155) occurs first, with later binding to TSP by the second region of CD36 (represented by polypeptide P93-110).

Prior binding of a ligand to TSP at the site to which polypeptide P139-155 binds causes induction of the other site on TSP and the binding of CD36 to the induced site (Panel B). Because the affinity for binding at the induced site is believed to be greater than at the other site, the net effect is augmentation of the TSP-CD36 interaction.

Binding of a ligand to the site at which polypeptide P93-110 binds, however, prevents CD36 from binding at that site (Panel C). Because the affinity of binding of CD36 to the other

TSP site is believed to be much lower, the net effect is inhibition of the interaction between TSP and CD36.

Although polypeptide ligands having amino acid sequences defined by SEQ ID NOs:1 and 2 are preferred for use in modulating the interactions between TSP and its receptors, those skilled in the art will appreciate that polypeptides for use in this invention might also contain more or fewer amino acid residues based on the known sequence of CD36, as long as function is not substantially impaired. They may also contain residues not normally present in the sequence of CD36, as shown in the Example below.

As explained above, the ligands of the invention encompass more than polypeptides and include other molecules such as antibodies as well. For example, a monoclonal antibody described below exhibited the same effects on TSP-CD36 interactions as did polypeptide P93-110. This antibody is characterized by about 5 fold greater binding to human TSP to which has been bound a polypeptide having an amino acid sequence defined by SEQ ID NO: 1, compared to TSP to which such a polypeptide has not been bound. This binding differential provides a convenient basis for screening hybridomas producing other antibodies having a similar specificity for binding TSP.

The ligands of this invention may be useful for modulating TSP-CD36 interactions in any process in which such interactions are known or can be shown to occur.

For example, as noted above, *Plasmodium falciparum*-infected erythrocyte adhesion to microvascular endothelial cells is believed to be mediated by infected cell recognition of CD36. There is also evidence that CD36 on tumor cells mediates adhesion to TSP [Asch *et al., J. Biol. Chem.* *266*:1740 (1991)]. The ligands of the invention which inhibit TSP-CD36 interactions may be used to block or reverse such adhesion.

There is also evidence that TSP-CD36 interactions play a role in stabilizing platelet aggregates [Asch *et al., J. Clin. Invest. 79*:1054 (1987); McGregor *et al., J. Biol. Chem. 264*:501 (1989)]. The ligands of the invention which inhibit TSP-CD36 interactions may be thus also be useful for reducing or preventing platelet aggregation. Such use of these ligands provides a novel approach to the design of anti-platelet and anti-thrombotic agents.

Current efforts are mainly focused on the disruption of the platelet $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) interaction with fibrinogen, using monoclonal antibodies or polypeptides based on the integrin sequence and Arg-Gly-Asp-containing analogs [Yasuda *et al., J. Clin. Invest. 81*:1284 (1988); Shebuski *et al., J. Biol. Chem. 264*:21550 (1989); Scarborough *et al., J. Biol. Chem. 266*:9359 (1991)]. Although such anti-platelet agents are functionally quite potent, they also have the potential to produce undesired bleeding, as evidenced by a marked prolongation of the bleeding time (Yasuda *et al., supra*; Shebuski *et al., supra*).

By interfering with the secondary phase of platelet aggregation while keeping the primary phase functionally intact, use of the ligands which inhibit TSP-CD36 interactions may provide a new therapeutic approach which minimizes the bleeding diathesis.

Finally, the use of the polypeptide ligands of this invention which augment TSP-CD36 interactions may be useful for enhancing platelet aggregation in situations where such aggregation is desirable. Such a situation arise in the treatment of patients with functional disorders or immune-mediated thrombocytopenia who cannot tolerate conventional platelet transfusions.

Currently, platelet transfusions are given to bleeding patients who lack platelets, and desmopressin (DDAVP) is given to those who have functionally defective platelets. In the case of

the former treatment, however, some patients are refractory due to an existing autoimmune disorder or will become refractory due to alloimmunization. In the case of DDAVP treatment, it is known that the effect is temporary and cannot be used on a long-term basis.

The polypeptide ligands which augment TSP-CD36 interactions in the case of platelets do not act by themselves but instead enhance ADP- and collagen-induced platelet aggregation in platelet-rich plasma.

The polypeptide ligands of the invention are synthesized by a suitable method such as by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. The polypeptides are preferably prepared by solid phase peptide synthesis as described by Merrifield, *J. Am. Chem. Soc. 85*:2149 (1963). The synthesis is carried out with amino acids that are protected at the alpha-amino terminus. Trifunctional amino acids with labile side-chains are also protected with suitable groups to prevent undesired chemical reactions from occurring during the assembly of the polypeptides. The alpha-amino protecting group is selectively removed to allow subsequent reaction to take place at the amino-terminus. The conditions for the removal of the alpha-amino protecting group do not remove the side-chain protecting groups.

The alpha-amino protecting groups are those known to be useful in the art of stepwise polypeptide synthesis. Included are acyl type protecting groups (e.g., formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups [e.g., benzyloxycarbonyl (Cbz), substituted benzyloxycarbonyl and 9-fluorenylmethyloxycarbonyl (Fmoc)], aliphatic urethane protecting groups (e.g., t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl) and alkyl type protecting groups (e.g.,

benzyl, triphenylmethyl). The preferred protecting group is Boc. The side-chain protecting groups for Tyr include tetrahydropyranyl, tert.-butyl, trityl, benzyl, Cbz, 4-Br-Cbz and 2,6-dichlorobenzyl. The preferred side-chain protecting group for Tyr is 2,6-dichlorobenzyl. The side-chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl and cyclohexyl. The preferred side-chain protecting group for Asp is cyclohexyl. The side-chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl and Cbz. The preferred protecting group for Thr and Ser is benzyl. The side-chain protecting groups for Arg include nitro, Tos, Cbz, adamantyloxycarbonyl and Boc. The preferred protecting group for Arg is Tos. The side-chain amino group of Lys may be protected with Cbz, 2-Cl-Cbz, Tos or Boc. The 2-Cl-Cbz group is the preferred protecting group for Lys.

The side-chain protecting groups selected must remain intact during coupling and not be removed during the deprotection of the amino-terminus protecting group or during coupling conditions. The side-chain protecting groups must also be removable upon the completion of synthesis, using reaction conditions that will not alter the finished polypeptide.

Solid phase synthesis is usually carried out from the carboxy-terminus by coupling the alpha-amino protected (side-chain protected) amino acid to a suitable solid support. An ester linkage is formed when the attachment is made to a chloromethyl or hydroxymethyl resin, and the resulting polypeptide will have a free carboxyl group at the C-terminus. Alternatively, when a benzhydrylamine or p-methylbenzhydrylamine resin is used, an amide bond is formed and the resulting polypeptide will have a carboxamide group at the C-terminus. These resins are commercially available, and their preparation has described by Stewart *et al.*, "Solid Phase

Peptide Synthesis" (2nd Edition), Pierce Chemical Co., Rockford, IL., 1984.

The C-terminal amino acid, protected at the side-chain if necessary and at the alpha-amino group, is coupled to the benzhydrylamine resin using various activating agents including dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide and carbonyldiimidazole. Following the attachment to the resin support, the alpha-amino protecting group is removed using trifluoroacetic acid (TFA) or HCl in dioxane at a temperature between 0° and 25°C. Dimethylsulfide is added to the TFA after the introduction of methionine (Met) to suppress possible S-alkylation. After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the required order to obtain the desired sequence.

Various activating agents can be used for the coupling reactions including DCC, N,N'-diisopropyl-carbodiimide, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and DCC-hydroxybenzotriazole (HOBt). Each protected amino acid is used in excess (>2.0 equivalents), and the couplings are usually carried out in N-methylpyrrolidone (NMP) or in DMF, CH_2Cl_2 or mixtures thereof. The extent of completion of the coupling reaction is monitored at each stage, e.g., by the ninhydrin reaction as described by Kaiser *et al.*, *Anal. Biochem.*, *34*:595 (1970). In cases where incomplete coupling is found, the coupling reaction is repeated. The coupling reactions can be performed automatically with commercially available instruments.

After the entire assembly of the desired polypeptide, the polypeptide-resin is cleaved with a reagent such as liquid HF for 1-2 hours at 0°C, which cleaves the polypeptide from the resin and removes all side-chain

protecting groups. A scavenger such as anisole is usually used with the liquid HF to prevent cations formed during the cleavage fom alkylating the amino acid residues present in the polypeptide. The polypeptide-resin may be deprotected with TFA/dithioethane prior to cleavage if desired.

Side-chain to side-chain cyclization on the solid support requires the use of an orthogonal protection scheme which enables selective cleavage of the side-chain functions of acidic amino acids (e.g., Asp) and the basic amino acids (e.g., Lys). The 9-fluorenylmethyl (Fm) protecting group for the side-chain of Asp and the 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group for the side-chain of Lys can be used for this purpose. In these cases, the side-chain protecting groups of the Boc-protected polypeptide-resin are selectively removed with piperidine in DMF. Cyclization is achieved on the solid support using various activating agents including DCC, DCC/HOBt or BOP. The HF reaction is carried out on the cyclized polypeptide-resin as described above.

Recombinant DNA methodology can also be used to prepare the polypeptides. The known genetic code, tailored if desired for more efficient expression in a given host organism, can be used to synthesize oligonucleotides encoding the desired amino acid sequences. The phosphoramidite solid support method of Matteucci *et al.* [*J. Am. Chem. Soc.* *103*:3185 (1981)], the method of Yoo *et al.* [*J. Biol. Chem.* *764*:17078 (1989)], or other well known methods can be used for such synthesis. The resulting oligonucleotides can be inserted into an appropriate vector and expressed in a compatible host organism.

The polypeptides of the invention can be purified using HPLC, gel filtration, ion exchange and partition chromatography, countercurrent distribution or other known methods.

The antibody ligands of the invention which inhibit TSP-CD36 interactions can be prepared against TSP using methods described below. Although polyclonal antiserum could in principle be used, monoclonal antibodies are preferred.

Antibodies can be produced by immunizing a host animal such as a rabbit, rat, goat, sheep, mouse, etc. with TSP. Preferably, one or more booster injections are given after the initial injection, to increase the antibody titer. Blood is then drawn from the animal and serum is prepared and screened by standard methods such as enzyme-linked immunosorbent assay (ELISA) using TSP as the antigen.

The immunogenicity of the TSP used for immunization can be increased by combination with an adjuvant and/or by conversion to a larger form prior to immunization. Suitable adjuvants for the vaccination of animals include but are not limited to Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); Freund's complete or incomplete adjuvant; mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecyl-ammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The polypeptides could also be administered following incorporation into liposomes or other microcarriers.

Monoclonal antibodies can be prepared using standard methods, e.g., as described by Kohler *et al.* [*Nature 256*:495 (1975); *Eur. J. Immunol. 6*:511 (1976)]. Essentially, an animal is immunized as described above to produce

antibody-secreting somatic cells. These cells are then removed from the immunized animal for fusion to myeloma cells.

Somatic cells with the potential to produce antibodies, particularly B cells, are suitable for fusion with a myeloma cell line. These somatic cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals. In the exemplary embodiment of this invention rat spleen cells are used, in part because these cells produce a relatively high percentage of stable fusions with mouse myeloma lines. It would be possible, however, to use human, mouse, rabbit, sheep, goat or other cells instead.

Specialized myeloma cell lines have been developed from lymphocytic tumors for use in hyridoma-producing fusion procedures [Kohler and Milstein, *Eur. J. Immunol. 6*:511 (1976); Shulman *et al.*, *Nature 276*:269 (1978); Volk *et al.*, *J. Virol. 42*:220 (1982)]. These cell lines have been developed for at least three reasons. The first is to facilitate the selection of fused hybridomas from unfused and similarly indefinitely self-propagating myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocytic tumor cells to produce their own antibodies. The purpose of using monoclonal techniques is to obtain fused hybrid cell lines with unlimited life spans that produce the desired single antibody under the genetic control of the somatic cell component of the hybridoma. To eliminate the production of tumor cell antibodies by the hybridomas, myeloma cell lines incapable of producing endogenous light or heavy immunoglobulin chains are used. A third reason for selection of these cell lines is for their suitability and efficiency for fusion.

Many myeloma cell lines may be used for the production of fused cell hybrids, including, e.g., P3X63-Ag8, P3X63-AG8.653, P3/NS1-Ag4-1 (NS-1), Sp2/0-Ag14 and S194/5.XXO.Bu.1. The P3X63-Ag8 and NS-1 cell lines have been described by Kohler and Milstein [*Eur. J. Immunol. 6*:511 (1976)]. Shulman *et al.* [*Nature 276*:269 (1978)] developed the Sp2/0-Ag14 myeloma line. The S194/5.XXO.Bu.1 line was reported by Trowbridge [*J. Exp. Med. 148*:313 (1979)].

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually involve mixing somatic cells with myeloma cells in a 10:1 proportion (although the proportion may vary from about 20:1 to about 1:1), respectively, in the presence of an agent or agents (chemical, viral or electrical) that promotes the fusion of cell membranes. Fusion methods have been described by Kohler and Milstein, *supra*, Gefter *et al.* [*Somatic Cell Genet. 3*:231 (1977)], and Volk *et al.* (*J. Virol. 42*:220 (1982)]. The fusion-promoting agents used by those investigators were Sendai virus and polyethylene glycol (PEG).

Because fusion procedures produce viable hybrids at very low frequency (e.g., when spleens are used as a source of somatic cells, only one hybrid is obtained for roughly every 1 x 10^5 spleen cells), it is essential to have a means of selecting the fused cell hybrids from the remaining unfused cells, particularly the unfused myeloma cells. A means of detecting the desired antibody-producing hybridomas among other resulting fused cell hybrids is also necessary.

Generally, the selection of fused cell hybrids is accomplished by culturing the cells in media that support the growth of hybridomas but prevent the growth of the unfused myeloma cells, which normally would go on dividing indefinitely. The somatic cells used in the fusion do not maintain long-term viability in *in vitro* culture and hence do not pose a problem. For

example, myeloma cells lacking hypoxanthine phosphoribosyl transferase (HPRT-negative) can be used. Selection against these cells is made in hypoxanthine/aminopterin/thymidine (HAT) medium, a medium in which the fused cell hybrids survive due to the HPRT-positive genotype of the spleen cells. The use of myeloma cells with different genetic deficiencies (drug sensitivities, etc.) that can be selected against in media supporting the growth of genotypically competent hybrids is also possible.

Several weeks are required to selectively culture the fused cell hybrids. Early in this time period, it is necessary to identify those hybrids which produce the desired antibody, so that they may subsequently be cloned and propagated. Generally, around 10% of the hybrids obtained produce the desired antibody, although a range of from about 1 to about 30% is not uncommon. The detection of antibody-producing hybrids can be achieved by any one of several standard assay methods, including enzyme-linked immunoassay and radioimmunoassay techniques which have been described in the literature [see, e.g., Kennet *et al.* (editors), Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses, pp. 376-384, Plenum Press, New York (1980)].

Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing cell lines, each cell line may be propagated in either of two standard ways. A suspension of the hybridoma cells can be injected into a histocompatible animal. The injected animal will then develop tumors that secrete the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propagated *in vitro* in laboratory culture vessels. The culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by

decantation, filtration or centrifugation, and subsequently purified.

Hybridomas producing monoclonal antibodies that can inhibit TSP-CD36 interactions are preferably identified by a two-stage screening process. First, hybridoma clones producing antibodies against TSP generally are identified by, e.g., ELISA, radioimmunoassay or gel electrophoresis/Western blotting, using TSP as the antigen. Secondly, clones thus identified are further analyzed for increased binding affinity for TSP in the presence of polypeptide P139-155 or another ligand that binds to TSP at the same site. As explained above, prior binding of polypeptide P139-155 to TSP causes induction of the site to which the inhibitory antibody ligands of the invention bind and about a five-fold increase in binding of the antibodies. Such increased binding of antibodies to TSP in the presence of the polypeptide can readily be detected and quantified, e.g., as described in the Example below.

Once a hybridoma producing the desired monoclonal antibody is obtained, techniques can be used to produce interspecific monoclonal antibodies wherein the binding region of one species is combined with a non-binding region of the antibody of another species [Liu *et al.*, *Proc. Natl. Acad. Sci.* USA *84*:3439 (1987)]. For example, the CDRs from a rodent monoclonal antibody can be grafted onto a human antibody, thereby "humanizing" the rodent antibody [Riechmann *et al.*, *Nature 332*:323 (1988)]. More particularly, the CDRs can be grafted into a human antibody variable region with or without human constant regions. Such methodology has been used, e.g., to humanize a mouse monoclonal antibody against the p55 (Tac) subunit of the human interleukin-2 receptor [Queen *et al.*, *Proc. Natl. Acad. Sci.* USA *86*:10029 (1989)].

Alternatively, human monoclonal antibodies can be prepared using the antigen disclosed herein and methods described by Banchereau *et al.* [*Science 251*:70 (1991)].

The present invention also encompasses antibody binding fragments such as Fab, $F(ab')_2$, Fv fragments, etc. The use and generation of fragments of antibodies is well known, e.g., Fab fragments [Tijssen, *Practice and Theory of Enzyme Immunoassays* (Elsevier, Amsterdam, 1985)], Fv fragments [Hochman *et al.*, *Biochemistry 12*:1130 (1973); Sharon *et al.*, *Biochemistry 15*:1591 (1976); Ehrlich *et al.*, U.S. Patent No. 4,355,023] and antibody half molecules (Auditore-Hargreaves, U.S. Patent No. 4,470,925).

Human TSP for use in producing the hybridomas and monoclonal antibodies of the invention can be prepared and purified by known methods, e.g., as described in the Example below.

Ligands other than polypeptides and antibodies or antibody fragments, such as cyclic peptides and non-peptide organic compounds, can be identified using the methods disclosed herein. For example, other ligands that are functionally equivalent to polypeptide P139-155 can be identified because they too, by binding to the same region on TSP, will augment TSP-CD36 interactions and enhance the binding of polypeptide P93-110 to TSP. Similarly, other ligands that are functionally equivalent to polypeptide P93-110 and the antibodies produced by hybridoma clone 7AIe can be identified because their binding to TSP will also be enhanced by prior binding of polypeptide P139-155, and they will inhibit TSP-CD36 interactions.

The ligands of the invention can be administered to a human patient requiring modulation of the interaction of TSP with platelets or cells bearing TSP receptors either directly in a buffered physiological solution or in the form of a pharmaceutical composition. Pharmaceutical compositions can

be prepared which contain effective amounts of one or more of the ligands and a physiologically acceptable carrier. Such carriers are well known to those skilled in the art (see, e.g., Remington's Pharmaceutical Sciences and U.S. Pharmacopeia: National Formulary, 1984, Mack Publishing Company, Easton, PA).

Administration may be by any suitable method, e.g., by parenteral administration, including intravenous, intraarterial, intraperitoneal, intramuscular and subcutaneous administration. Administration may be by bolus injection, continuous infusion, sustained release from implanted delivery systems [Urquhart *et al., Ann. Rev. Pharmacol. Toxicol. 24*:199 (1984)] or by other known methods.

In general, the polypeptide and antibody ligands will be administered at a dosage of from about 1 µg/kilogram body weight/day to about 50 mg/kilogram body weight/day, with a preferred dosage of from about 1 mg/kilogram body weight/day to about 10 mg/kilogram body weight/day.

Determination of the proper dosage of a ligand for a particular situation is within the skill of the art. Generally, treatment is initiated with smaller dosages that are less than optimum. Thereafter, the dosage is increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day if desired.

The amount and frequency of administration of the ligands of the invention and the pharmaceutically acceptable salts thereof will be regulated according to the judgment of the attending clinician, taking into account such factors as age, condition and size of the patient and severity of the condition(s) being treated.

EXAMPLE

In the following non-limiting Example used for the purpose of illustration, percentages for solids in solid mixtures, liquids in liquids and solids in liquids are expressed on a wt/wt, vol/vol and wt/vol basis, respectively, unless otherwise indicated.

Polypeptide Synthesis

The polypeptide ligands having amino acid sequences defined by SEQ ID NO: 1 and SEQ ID NO: 2 will for convenience be referred to below as polypeptides P139-155 and P93-110, respectively. These polypeptides, together with eight others having amino acid sequences defined by SEQ ID NOs: 3-10, had sequences based upon subsequences of CD36.

All of the polypeptides were prepared by solid phase synthesis using an Applied Biosystems Model 430 peptide synthesizer and t-boc chemistry. Cleavage of the peptides from the resin was performed with anhydrous HF at -5°C in the presence of 10% anisole. The peptides were precipitated with ether, dissolved in 0.25 M acetic acid and lyophilized.

A cysteine and a tyrosine residue were added to each peptide if the CD36 subsequence upon which the peptide was based did not contain these residues, to allow the peptides to be conjugated to carrier proteins or iodinated if desired. The locations of these residues within the peptides to which they were added were as follows:

Peptide SEQ ID NO	Residue (Number)
1	Cys (1)
2	Cys (19)
3	Tyr (1); Cys (20)
4	Cys (1); Tyr (2)
5	Cys (1)
6	Cys (1)
8	Cys (1)
9	Cys (1); Tyr (2)
10	Cys (1)

Collectively, the synthesized peptides comprised about 40% of the extracellular domain of CD36.

Protein Purification

CD36 was purified from Triton X-114-solubilized human platelet membrane extracts by FPLC anion exchange chromatography essentially as described by McGregor *et al.* [*J. Biol. Chem. 264*:501 (1989)] except that wheat germ agglutinin affinity chromatography was performed instead of FPLC gel filtration as the final purification step, as described by Tandon *et al.* [*J. Biol. Chem. 264*:7570 (1989)]. TSP was purified from thrombin-induced human platelet releasates, essentially as described by Leung *et al.* [*J. Clin. Invest. 70*:542 (1982)] and Clezardin *et al.* [*Eur. J. Biochem. 154*:95 (1986)].

Monoclonal Antibody Production

A three-month old female Lewis rat was immunized intraperitoneally (i.p.) with a 0.25 ml solution of 100 purified human TSP, emulsified with an equal volume of complete Freund's adjuvant. Three weeks after the initial immunization, the rat was boosted i.p. with 50 µg of TSP emulsified with incomplete Freund's adjuvant. This booster was repeated three weeks later. Three weeks thereafter, after confirming serum reactivity by ELISA on whole TSP, the rat was boosted again.

Three days after the final booster injection the rat was sacrificed, blood was collected, and the spleen was removed for fusion.

Spleen cells were fused with mouse myeloma cells, P3X63-Ag8.653 (ATCC CRL 1580), in a 1:1 ratio using polyethylene glycol (PEG). The cell suspension (3.5×10^5 cells/ml in HAT medium) was distributed into 40 96-well plates.

Six days after plating the fusion products, the HAT medium was replaced with HT medium. Hybridoma supernatants were tested in a primary screen for their ability to bind to human TSP two, three and four weeks after fusion. Cells in positive wells were then expanded, and the medium was replaced with RPMI medium containing penicillin/streptomycin (Pen-Strep) and 10% fetal calf serum (FCS).

The primary screen was a standard ELISA in which plates having U-shaped wells were coated overnight at 4°C with 50 µl of a 10 µg/ml solution of TSP in 1X TBS (Tris-buffered saline; 20 mM Tris, 0.15 M NaCl, pH 7.4) with 2% CaCl. The plates were washed twice with TBS containing 2% CaCl and 0.05% TWEEN 20® (polyoxyethylenesorbitan monolaurate), after which 100 µl of hybridoma supernatant were added to each well and the plates were incubated at room temperature for two hours.

The wells were washed twice as described above, and 50 µl of a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-rat IgG in TBS/CaCl were added to each well and incubated for one hour at room temperature. The wells were again washed twice, and 50 µl of BIORAD alkaline phosphatase substrate solution (a buffered solution of p-nitrophenyl phosphate) were added to each well. After a 15 minute incubation at room temperature, the plates were read at 405 nm. About 200 hybridoma cell lines testing positive in the primary screen were thus identified.

The positive hybridomas were then evaluated in a secondary ELISA screen. In this screen U-shaped wells were coated overnight at 4°C as described by Leung *et al.* [*J. Clin. Invest. 70*:542 (1982)] with 200 µl of 5 µg/ml human TSP in bicarbonate coating buffer (15 mM Na_2CO_3, 34.8 mM $NaHCO_3$, pH 9.6). The plates were then blocked with 5% bovine serum albumin in TBS for 30 minutes at room temperature, and the wells were washed twice with TBS containing 2 mM $CaCl_2$ and 0.05% TWEEN 20®.

One hundred microliter aliquots of the hybridoma supernatants were combined with 100 µl of 2X Tris/0.05% TWEEN 20®, with and without polypeptide P139-155 to a final concentration of 10 µg/ml. The resulting solutions were added to the washed wells, and the plates were incubated for four hours at 37°C. After washing the wells twice as described above, 200 µl of a 1:500 dilution of alkaline phosphatase-conjugated goat anti-rat IgG were added to each well. The plates were incubated for one hour at 37°C, developed and then read as described above.

Cells were selected from the secondary screen which secreted antibodies that bound at least about 4 times more to the TSP in the presence of polypeptide P139-155 than in the absence of the polypeptide. Three hybridoma cell lines were thus identified, one of which was designated 7A.

The hybridoma cell lines testing positive in the secondary screen were then subcloned by limiting dilution. This was accomplished by seeding a solution containing 360 cells/well in RPMI/10% FCS/Pen-Strep into the top row of a microtiter plate, and then carrying out serial 2-fold dilutions down the plate. The bottom row would have been predicted to have 0.25 cell/well on average, as a result of this procedure.

After two weeks, wells containing single colonies (as determined by visual inspection at 4X and 10X) were expanded. As a result of such cloning of hybridoma cell line 7A, three clones were isolated, two of which were designated 7AIe and 7AIh.

Protein and Polypeptide Iodination

TSP, CD36 and the polypeptides having amino acid sequences defined by SEQ ID NOs: 1, 2 and 5 were radiolabeled with ^{125}I using the IODOBEAD® method (Pierce Chemical Co.), essentially as described by McGregor *et al.* [*J. Biol. Chem.* 264:501 (1989)]. The labeled peptides were separated from free ^{125}I by SEPHADEX G-25® gel filtration chromatography (1.3 x 25 cm) and eluted with phosphate buffered saline (PBS) at a flow rate of 0.5 ml/minute.

Immunoadsorption of Labeled CD36 and Polypeptide P139-155 by Antibody OKM5

^{125}I-CD36 or ^{125}I-P139-155 (1 x 10^6 cpm/sample) was incubated with 3 µg/sample of antibody OKM5 (Ortho Pharmaceuticals) or a control monoclonal antibody against an unrelated antigen (designated antibody 1F5) for 2 hours at room temperature in the presence or absence of 10 µg/sample of the various synthetic polypeptides. Protein A SEPHAROSE® (15 µl/sample) that had been pre-washed in PBS with 0.5% bovine serum albumin (BSA) was added, and after 1 hour of incubation at room temperature, the SEPHAROSE® was washed three times with Tris-Tween buffer [20 mM Tris-HCl, 0.05% TWEEN 20®, pH 7.4] and the bound immune complexes were eluted by boiling with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [Laemmli, *Nature* 227:680 (1970)].

The eluted immune complexes were analyzed by SDS-PAGE (Laemmli, *supra*) and autoradiography, with equal amounts of counts per sample loaded in the gels. 7.5% SDS-PAGE

gels were used except for analysis of the labeled polypeptide, where 20% gels were used. The bands in the gels were located by staining with Coomassie blue.

The results showed that by itself antibody OKM5 immunoadsorbed ^{125}I-CD36 and that only polypeptide P139-155 consistently blocked such immunoadsorption. This inhibitory effect was specific, because the polypeptide did not block immunoadsorption of ^{125}I-albumin by a monoclonal antibody against albumin.

The specific inhibition of CD36 immunoadsorption by OKM5 by P139-155 suggested that the polypeptide might represent part of the CD36 epitope recognized by the antibody. Alternatively, it was possible that the polypeptide bound to CD36, thereby causing a conformational change which indirectly affected the epitope against which OKM5 was directed.

To determine which was the case, the capacity of OKM5 to directly immunoadsorb ^{125}I-P139-155 was investigated. The result was that OKM5 did in fact directly immunoadsorb the labeled P139-155 polypeptide but not other polypeptides, thereby ruling out an indirect, conformational effect on CD36. This specific immunoadsorption was inhibited by excess unlabeled P139-155.

Binding of Thrombospondin to Polypeptide P139-155

Because antibody OKM5 has been shown to block interaction between TSP and CD36 and polypeptide P139-155 represents part of the OKM5 epitope, it was possible that the polypeptide represented part of the binding site on CD36 for TSP. To determine whether this was so, ELISA was carried out essentially as described by Leung *et al.* [*J. Clin. Invest. 70*:542 (1982)] using surface-immobilized P139-155, TSP and a monoclonal antibody against TSP designated antibody P10^{35} (available from AMAC).

Briefly, polypeptide P139-155 was coated onto microtiter wells at a concentration of 10 μg/ml in bicarbonate coating buffer (15 mM Na_2CO_3, 34.8 mM $NaHCO_3$, pH 9.5) by incubating overnight at 4°C. The wells were washed with Tris-Tween buffer, and nonspecific sites were blocked with Tris-Tween/0.5% BSA. TSP (1, 2, 4 and 8 μg/ml) was added in Tris-Tween buffer and incubated for 3 hours at 37°C. Binding of TSP to polypeptide P139-155 was monitored by the sequential addition of anti-TSP antibody $P10^{35}$ (10 μg/ml) followed by goat anti-mouse IgG conjugated with alkaline phosphatase (1:7,500) and enzyme substrate. Absorbance at 405 nm was measured in a TITERTEK® plate reader.

It was found that TSP bound to the immobilized polypeptide in a dose-dependent, saturable manner, but not to several of the other synthetic polypeptides. To further confirm the specificity of the binding of TSP to immobilized polypeptide P139-155, increasing amounts of soluble P139-155 were added during a binding assay carried out as described above. It was thereby found that the binding of TSP to immobilized polypeptide P139-155 was inhibited 90% by the addition of 80 μg/ml soluble P139-155.

The results thus suggested that polypeptide P139-155 represents part of the CD36 molecule involved in TSP-CD36 binding.

Augmentation and Inhibition of the Binding of CD36 to Thrombospondin

The synthetic polypeptides described above were examined for possible effects on the binding of ^{125}I-CD36 to surface-immobilized TSP. Briefly, TSP (5 μg/ml) was immobilized on microtiter wells and increasing amounts of soluble ^{125}I-CD36 (specific radioactivity ≈ 600,000 cpm/μg) were added in the presence or absence of 20 μg/ml of each of the polypeptides in a binding assay carried out essentially as

described above. The only effects on the binding of the labeled CD36 to the immobilized TSP were observed in the presence of polypeptides P139-155 and P93-110. The results are shown in Fig. 2.

Because, as described above, polypeptide P139-155 appears to represent the part of the CD36 molecule involved in TSP-CD36 binding, it would be expected that by binding to TSP, the polypeptide would block the site at which TSP binds to CD36 and thereby inhibit the binding of the two proteins. Yet the data of Fig. 2 (upper curve) show just the opposite effect. Polypeptide P139-155 instead augmented or enhanced the binding of CD36 to TSP.

The binding of ^{125}I-CD36 to immobilized TSP was augmented by 68.2 ± 7.9% (mean ± SEM, n=15) in the presence of soluble polypeptide P139-155 at a concentration of 10 µg/ml. Seven other control polypeptides had no significant effect on CD36 binding (n=12). This result was repeatedly observed, in five separate experiments.

In contrast, polypeptide P93-110 (Fig. 2, lower curve), which also specifically binds to TSP but has no effect on the immunoprecipitation of CD-36 by antibody OKM5, did inhibit the binding of ^{125}I-CD36 to immobilized TSP. The degree of inhibition was 58 ± 2.5% (mean ± SEM, n=9) in the presence of soluble polypeptide P93-110 at a concentration of 5 µg/ml and 69.7 ± 5.7% (mean ± SEM, n=18) at a concentration of 10 µg/ml.

The results shown in Fig. 2 represent the average of triplicate determinations obtained in one of two similar experiments.

Similar inhibitory effects have been produced by the monoclonal antibodies produced by hybridoma cell line 7A and subclones thereof such as 7AIe and, to a much lesser degree,

7AIh. These two antibodies, bound to different sites on TSP and were shown to vary substantially in inhibitory potency.

In binding studies carried out as described above, it was found that antibodies from subclone 7AIe in the presence of polypeptide P139-155 significantly augmented the binding of ^{125}I-CD36 to TSP. In the presence of the polypeptide, 11.06 ± 0.85 ng/well of the labeled CD36 bound; in the absence of the polypeptide, only 4.36 ± 2.34 ng/well bound (mean±SD, n=6).

In the absence of polypeptide P139-155, the antibodies from subclones 7AIe and 7AIh both partially inhibited the binding of ^{125}I-CD36 to TSP. When the polypeptide was present, however, the marked inhibition of binding seen with antibodies from subclone 7AIe was not produced by antibodies from subclone 7AIh.

These results indicate that after induction of the high affinity CD36 binding site on TSP by polypeptide P139-155, the enhanced binding and occupancy of the epitope by 7AIe antibodies blocked high affinity binding of TSP for CD36. This suggests that the 7AIe epitope is critically involved in the high affinity binding site. In contrast, 7AIh antibodies evidently bound to a different site on TSP which partially interfered with baseline (without P139-155) binding, probably due to steric hindrance. This partial blockage, however, was completely eliminated in the presence of polypeptide P139-155, with induction of the high affinity binding site.

Effects On Platelet Aggregation

To determine whether the contrasting effects of polypeptides P93-110 and P139-155 on the binding of CD36 to TSP could be observed in a more physiological system, the polypeptides were examined for effects on human platelets.

Citrated platelet-rich human plasma was prepared and then incubated with polypeptides P139-155 and P93-110 at 37°C and at 1,000 rpm in a Chrono-Log two-channel aggregometer as described by Leung [J. Clin. Invest. *74*:1764 (1984)]. The platelets were then activated by the addition of ADP or collagen (Chrono-Log Corp., Havertown, PA), and aggregation was monitored as increased light transmission.

The results of a representative experiment are shown in Fig. 3, in which it can be seen that polypeptide P139-155 at a concentration of 30 μg/ml augmented platelet aggregation induced by 5 μM ADP (Panel A) and 1.2 μg/ml collagen (Panel B). Polypeptide P93-110 at a concentration of 25 μg/ml partially inhibited platelet aggregation induced by 1.4 μg/ml collagen (Panel C). Polypeptides designated P35-53, P208-224 and P313-329 having amino acid sequences defined by SEQ ID NOs: 3, 5 and 7, respectively, served as controls. The observed results were quantified by determining the areas under the aggregation tracings by planimetry.

As shown in Panel A, polypeptide P139-155 did not induce spontaneous platelet aggregation in the platelet-rich plasma but augmented aggregation induced by ADP by 44.5 ± 10.7% (mean ± SEM, n=11). This polypeptide also augmented collagen-induced aggegation by 101 ± 17.3% (mean ± SEM, n=5), with an increase in the size of the platelet aggregates as evidenced by increased amplitude in the aggregation tracing and by direct visual examination, compared to control P35-53 (Panel B). In contrast, as shown in Panel C, polypeptide P93-110 produced an inhibitory effect on collagen-induced platelet aggregation, reducing aggregation by 71.2 ± 10.7% (mean ± SEM, n=7).

Similar inhibitory effects have been produced by the monoclonal antibodies produced by hybridoma cell line 7A and subclone 7AIe. Data produced as described above using

antibodies from subclone 7AIe instead of the polypeptides are shown in Fig. 4, in which the amount of antibodies used is shown to the right of each tracing.

As is evident from Fig. 4, increasing amounts of antibodies from subclone 7AIe produced concentration-dependent increasing inhibition of platelet aggregation. In contrast, antibodies from subclone 7AIh had little effect, even at a concentration at which 7AIe antibodies produced almost complete inhibition.

Induction of Polypeptide P93-110 Binding to Thrombospondin

To further investigate the effects of the P139-155 polypeptide on TSP, the binding of labeled polypeptide P93-110 to immobilized TSP was measured, both in the presence and absence of polypeptide P139-155.

This was accomplished by immobilizing TSP on microtiter wells as described above and then adding increasing concentrations of ^{125}I-P93-110 (specific radioactivity 49,000 cpm/μg) in the presence or absence of a 4 μg/ml concentration of polypeptide P139-155 or of control polypeptide P208-224 or P228-242 (having amino acid sequences defined by SEQ ID NOs: 5 and 6, respectively). The plate was incubated for 3 hours at 37°C. After washing three times with Tris-Tween buffer, the bound ^{125}I-P93-110 was solubilized by SDS and measured by gamma counting, with the results shown in Fig. 5.

As shown in Fig. 5, ^{125}I-P93-110 by itself did not bind appreciably to the immobilized TSP. In the presence of unlabeled polypeptide P139-155, however, there was a marked enhancement of ^{125}I-P93-110 binding. This augmentation in binding by P139-155 was specific, because the control polypeptides at the same concentration had little or no effect.

Further binding studies showed that 7AIe antibodies completely blocked the binding of ^{125}I-labeled polypeptide P93-110 to TSP in the presence of polypeptide P139-155. In contrast, 7AIh antibodies showed no such blocking effect, again suggesting that these antibodies did not bind directly to the critical high affinity binding site on TSP.

Taken together, the foregoing results suggest that the binding sites on TSP for 7AIe antibodies and polypeptide P93-110 may be identical, or sterically related to each other.

Identification of the Induced TSP Binding Site

As noted above, the sequence Ser-Val-Thr-Cys-Gly (see also SEQ ID NO: 14) has been identified as a cell adhesive motif in TSP having homology to the maralia circumsporozoite protein. To determine whether this motif sequence was involved in the high affinity CD36 binding site induced by the binding of polypeptide P139-155 to TSP, a polypeptide having an amino acid sequence corresponding to that of residues 487 to 498 of the known sequence of TSP which contained the motif sequence was synthesized as described above, together with two polypeptides containing the motif amino acid residues but in scrambled order. The amino acid sequences of these polypeptides are defined in the Sequence Listing by SEQ ID NOs: 11 (correct sequence) and 12 and 13 (scrambled sequences).

Microtiter wells were coated with one of the polypeptides as described above, at a concentration of 10 μg/ml. An unrelated control antibody or antibodies from subclone 7AIe or 7AIh were then added at a concentration of 8 μg/ml to the wells at 37°C, and the plate was incubated for 3 hours. Following the incubation, the plate was washed to remove unbound antibodies, and the bound antibodies were detected with goat anti-rat IgG conjugated with alkaline phosphatase. The results represented the mean of two experiments, each done in triplicate.

It was found that antibodies from subclone 7AIe bound significantly to the polypeptide containing the motif sequence, compared to the scrambled polypeptides (ΔA_{405}/min=12.64 x 10^{-3} vs 4.76 - 4.96 x 10^{-3}), while antibodies from subclone 7AIh and the unrelated control antibody showed little binding (ΔA_{405}/min=4.09 - 4.95 x 10^{-3}).

Binding studies carried out as described above showed that ^{125}I-labeled polypeptide P93-110 bound specifically to the immobilized motif-containing polypeptide (9.89 ng/well) but not to the immobilized scrambled polypeptides (2.41-2.54 ng/well). In contrast, ^{125}I-labeled polypeptide P139-155 did not bind specifically to any of the immobilized polypeptides.

In view of the foregoing results, it is likely that the previously-identified motif sequence is located in the high affinity CD36 binding site induced by the binding of polypeptide P139-155 to TSP.

Hybridoma Deposits

Hybridoma clones 7AIe and 7AIh were deposited April 29, 1992 with the American Type Culture Collection (ATCC), Rockville, MD, under Accession Nos. HB 11033 and HB 11034, respectively. These deposits were made under the conditions provided by the ATCC's agreement for Culture Deposit for Patent Purposes, which assures that the deposits will be made available to the U.S. Commissioner of Patents and Trademarks pursuant to 35 U.S.C. § 122 and 37 C.F.R. § 1.14 and will be made available to the public upon issue of a U.S. patent, and which requires that the deposits be maintained. Availability of the deposited clones is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

Many modifications and variations of this invention can be made without departing from its spirit and scope, as will become apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Howard, Russell J.
Leung, Lawrence L.K.

(ii) TITLE OF INVENTION: Modulation of Thrombospondin-CD36 Interactions

(iii) NUMBER OF SEQUENCES: 14

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(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: Apple Macintosh

(C) OPERATING SYSTEM: Macintosh 6.0.5

(D) SOFTWARE: Microsoft Word 4.00B

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Cys Asn Leu Ala Val Ala Ala Ala Ser His Ile Tyr Gln Asn Gln Phe
1 5 10 15
Val Gln

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Tyr Arg Val Arg Phe Leu Ala Lys Glu Asn Val Thr Gln Asp Ala Glu
1 5 10 15
Asp Asn Cys

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Tyr Gln Lys Thr Ile Lys Lys Gln Val Val Leu Glu Glu Gly Thr Ile
1 5 10 15
Ala Phe Lys Cys
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Cys Tyr Ile Asn Lys Ser Lys Ser Ser Met Phe Gln Val Arg Thr Leu
1 5 10 15

Arg Glu Leu

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Cys Ala Asp Gly Val Tyr Lys Val Phe Asn Gly Lys Asp Asn Ile Ser
1 5 10 15
Lys Val

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Cys Asp Thr Tyr Lys Gly Lys Arg Asn Leu Ser Tyr Trp Glu Ser His
1 5 10 15

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Cys Thr Glu Lys Ile Ile Ser Lys Asn Cys Thr Ser Tyr Gly Val Leu
1 5 10 15
Asp

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Cys Lys Glu Gly Arg Pro Val Tyr Ile Ser Leu Pro His Phe Leu Tyr
1 5 10 15
Ala Ser

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Cys Tyr Val Ser Glu Pro Ile Asp Gly Leu Asn Pro Asn Glu Glu Glu
1 5 10 15
His Arg Thr

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Cys Val Lys Pro Ser Glu Lys Ile Gln Val Leu Lys Asn Leu Lys Arg
1 5 10 15
Asn Tyr

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ser Val Thr Cys Gly Gly Gly Val Gln Lys Arg Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Lys Ser Gly Thr Arg Gly Gln Ser Gly Val Cys Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 12 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Val Ile Asp Gly Ser Ile Cys Arg Gly Thr Thr Val
1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
Ser Val Thr Cys Gly
1               5
```

WHAT IS CLAIMED IS:

1. A ligand which selectively binds to a region of thrombospondin that specifically binds to a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

2. A ligand which selectively binds to a region of thrombospondin, the presence of which region is induced by the binding to thrombospondin of a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

3. A method for augmenting thrombospondin-mediated effects comprising contacting thrombospondin in the presence of platelets or cells bearing receptors for thrombospondin with an effective amount of a ligand which selectively binds to a region of thrombospondin that specifically binds to a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

4. The method of claim 3 in which the thrombospondin is contacted in the presence of platelets.

5. A method for inhibiting thrombospondin-mediated effects comprising contacting thrombospondin in the presence of platelets or cells bearing receptors for thrombospondin with an effective amount of a ligand which selectively binds to a region of thrombospondin, the presence of which region is induced by the binding to thrombospondin of a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

6. The method of claim 5 in which the thrombospondin is contacted in the presence of platelets.

7. The ligand or method of any one of claims 1, 3 or 4 in which the ligand is a polypeptide.

8. The ligand or method of claim 7 in which the ligand is a polypeptide having an amino acid sequence defined by SEQ ID NO: 1.

9. The ligand or method of any one of claims 2, 5 or 6 in which the ligand is a monoclonal antibody.

10. The ligand or method of claim 9 in which the ligand is a monoclonal antibody produced by hybridoma clone 7AIe that binds to a region of thrombospondin containing an amino acid sequence defined by SEQ ID NO: 14.

11. The ligand or method of any one of claims 2, 5 or 6 in which the ligand is a polypeptide.

12. The ligand or method of claim 11 in which the ligand is a polypeptide having an amino acid sequence defined by SEQ ID NO: 2.

13. A pharmaceutical composition comprising a ligand of either claim 1 or claim 2 and a pharmaceutically acceptable carrier.

14. A method for making a pharmaceutical composition comprising admixing a ligand of either claim 1 or claim 2 with a pharmaceutically acceptable carrier.

15. Hybridoma clones 7AIe and 7AIh, deposited with the American Type Culture Collection under Accession Nos. HB 11033 and HB 11034, respectively.

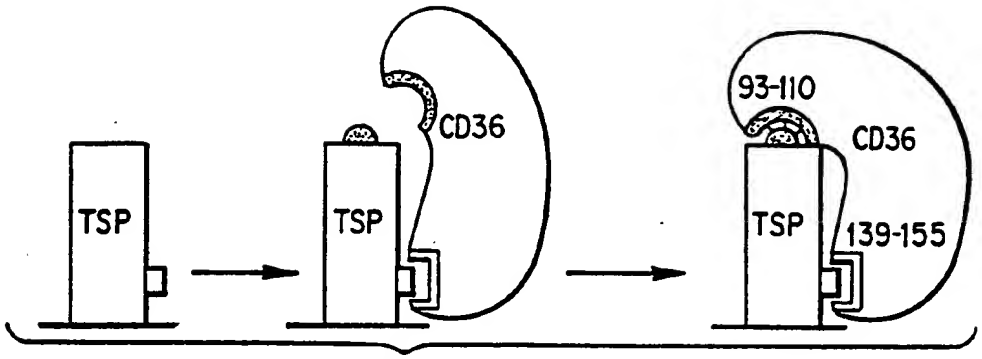

FIG. 1A

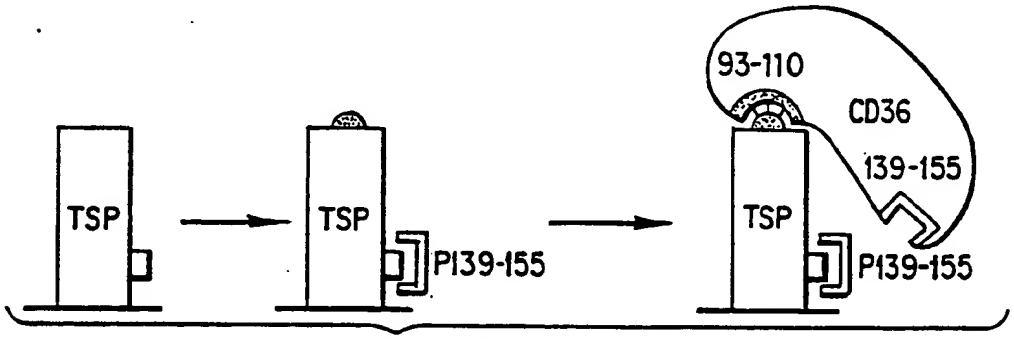

FIG. 1B

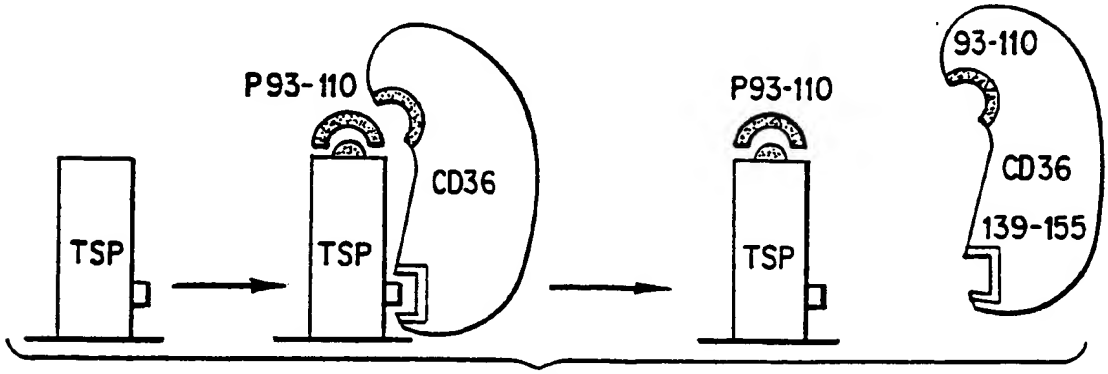

FIG. 1C

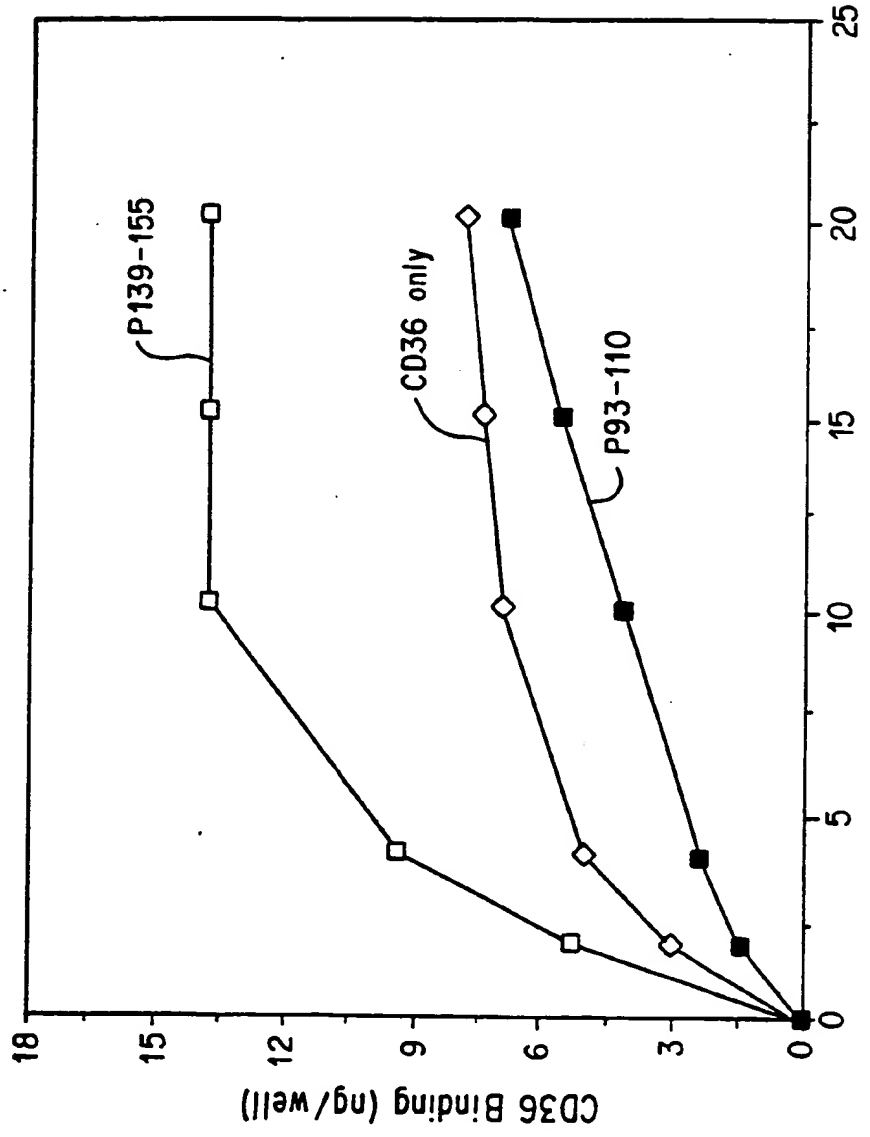
CD36 Binding (ng/well)
0
3
6
9
12
15
18
CD36 (µg/ml)
0
5
10
15
20
25
P139-155
CD36 only
P93-110

FIG. 2

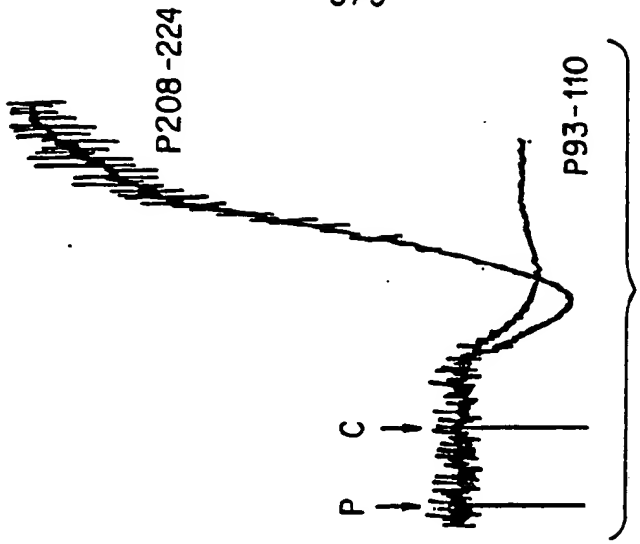

FIG. 3C

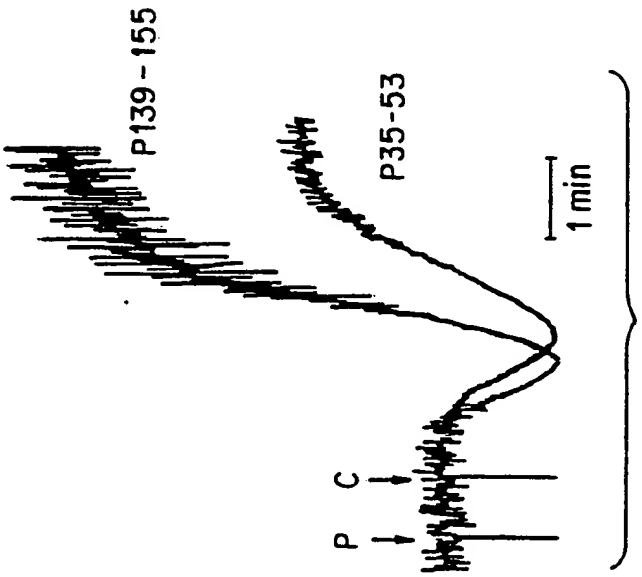

FIG. 3B

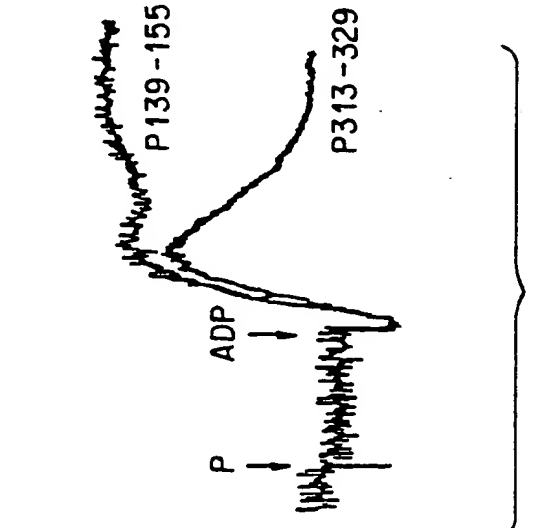

FIG. 3A

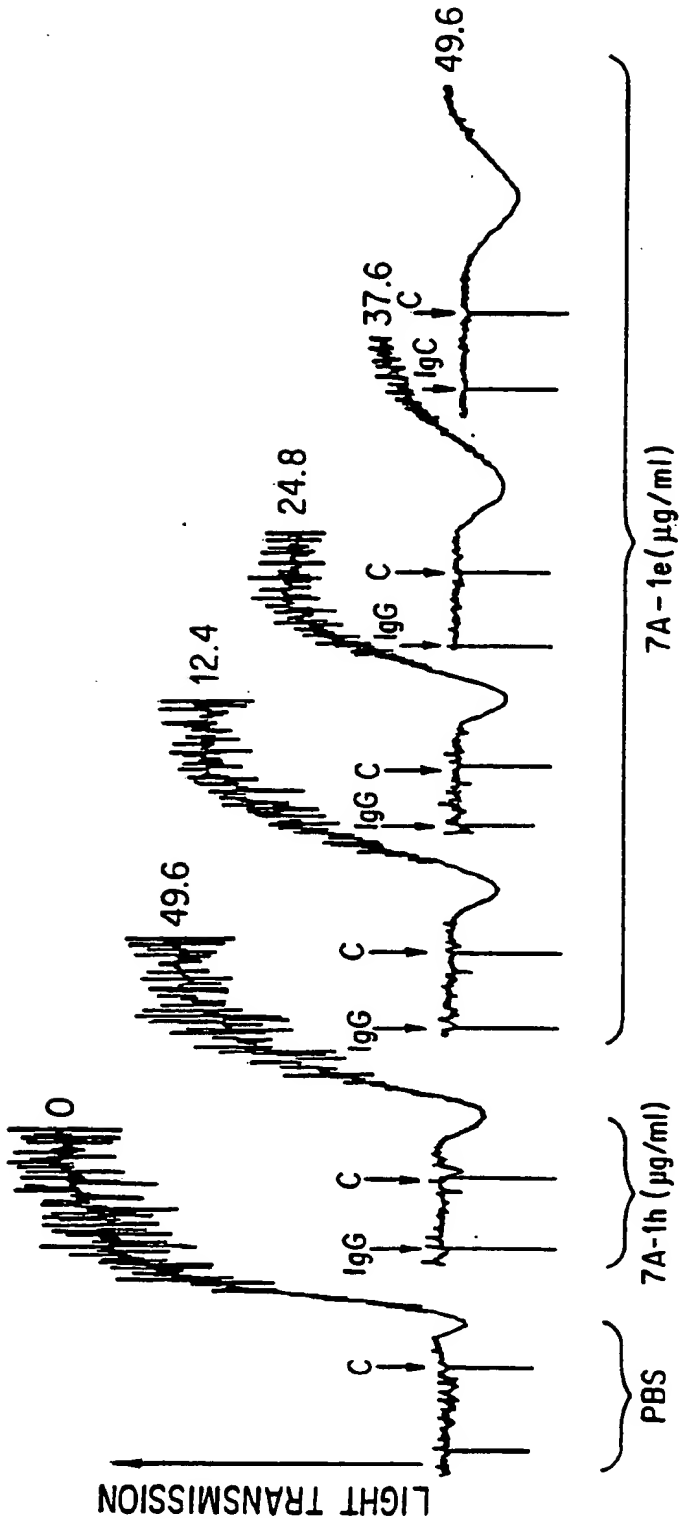
LIGHT TRANSMISSION
PBS
7A-1h (μg/ml)
7A-1e(μg/ml)
C
IgG
0
49.6
12.4
24.8
37.6
49.6

FIG. 4

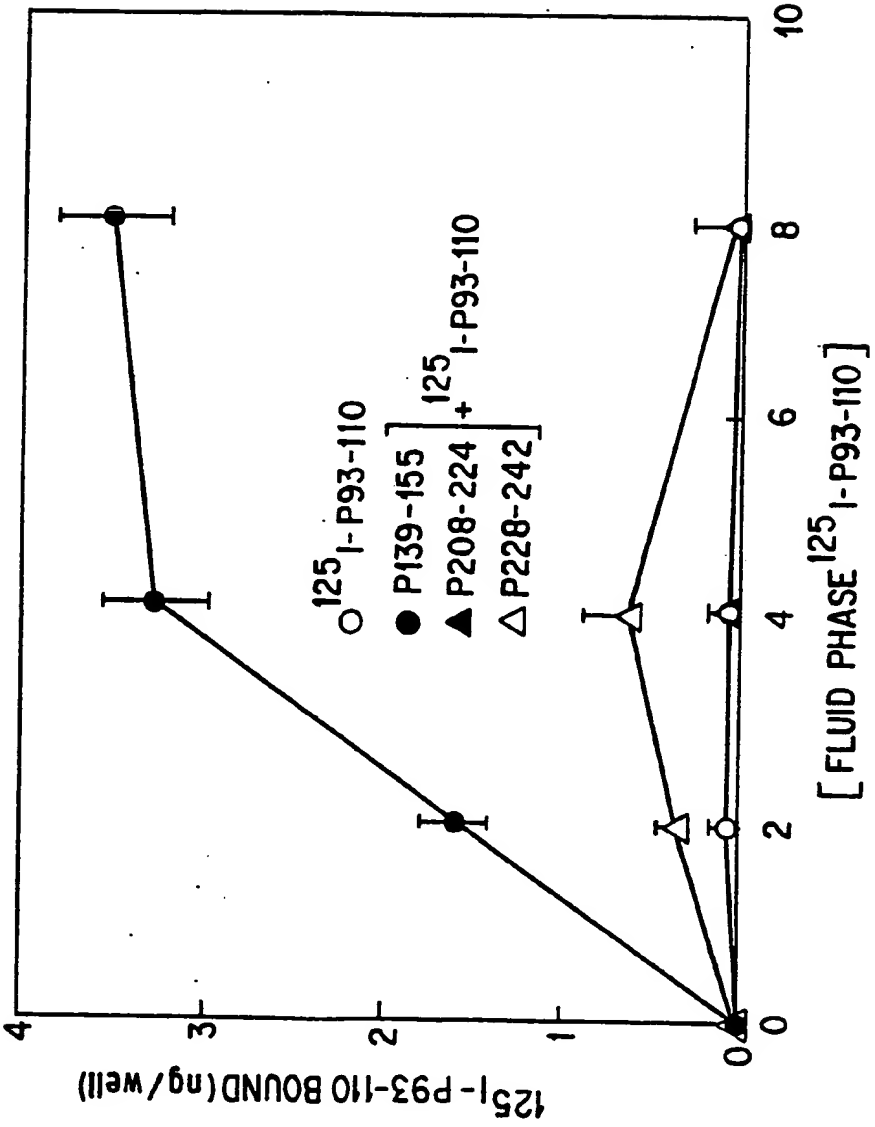
^{125}I-P93-110 BOUND (ng/well)
0
1
2
3
4
0
2
4
6
8
10
[FLUID PHASE ^{125}I-P93-110] (ug/ml)
○ ^{125}I-P93-110
● P139-155
▲ P208-224
△ P228-242
+ ^{125}I-P93-110

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/03748

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K7/08; C12P21/08; C12N5/20; A61K37/02
A61K39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[10]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,9 201 049 (THE GENERAL HOSPITAL CORPORATION, US) 23 January 1992 see example IX ---	1-8,11, 12
X	CELL vol. 58, 4 July 1989, CAMBRIDGE, NA US pages 95 - 101 OQUENDO, P. ET AL.; 'CD36 directly mediates cytoadherence of plasmodium falciparum parasitized erythrocytes.' see page 98, column 1, line 1 - page 99, column 1, line 32; figure 4 ---	1-8,11, 12
Y	EP,A,0 478 101 (THE MEDICAL COLLEGE OF PENNSYLVANIA) 1 April 1992 see page 14, line 55 - page 15, line 30; claims 3,15 ---	9,10
	-/--	

[10] Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26 AUGUST 1993	14 -09- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NAUCHE S.A.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 182, no. 3, 14 February 1992, DULUTH, MINNESOTA US pages 1208 - 1217 ASCH AS;SILBIGER S;HEIMER E;NACHMAN RL; 'Thrombospondin sequence motif (CSVTCG) is responsible for CD36 binding.' See the abstract	9,10
Y	EP,A,0 443 404 (THE MEDICAL COLLEGE OF PENNSYLVANIA) 28 August 1991 see page 10, line 55 - page 11, line 25	9,10
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 3, 1991, BALTIMORE US pages 1740 - 1745 ASCH AS;TEPLER J;SILBIGER S;NACHMAN RL; 'Cellular attachment to thrombospondin. Cooperative interactions between receptor systems.'	1-15
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 267, no. 25, 5 September 1992, BALTIMORE US pages 18244 - 18250 LEUNG LL;LI WX;MCGREGOR JL;ALBRECHT G;HOWARD RJ; 'CD36 peptides enhance or inhibit CD36-thrombospondin binding. A two-step process of ligand-receptor interaction.' see the whole document	1-15

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9303748
SA 73734

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 26/08/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9201049	23-01-92	AU-A-	8528691	04-02-92
		EP-A-	0551301	21-07-93
EP-A-0478101	01-04-92	US-A-	5190920	02-03-93
		CA-A-	2052022	25-03-92
		JP-A-	4288020	13-10-92
EP-A-0443404	28-08-91	US-A-	5190918	02-03-93
		US-A-	5200397	06-04-93